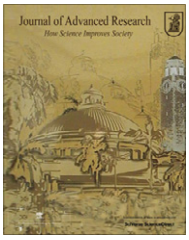
Journal of Advanced Research (2012) 3, 35–43

Cairo University

Journal of Advanced Research



ORIGINAL ARTICLE

The crude plant juices of desert plants as appropriate culture media for the cultivation of rhizospheric microorganisms

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Received 26 October 2010; revised 22 December 2010; accepted 4 March 2011
Available online 13 April 2011

KEYWORDS

Desert plants;
Plant juices;
Rhizospheric microorganisms;
Diazotrophs;
Culture media;
North Sinai

Abstract The exclusive use of plant juices, not as a mere supplement to synthetic culture media, for culturing rhizospheric microorganisms (RMO) is introduced here. Juices were prepared from desert (*Mesembryanthemum crystallinum* L., *Zygophyllum album* L., *Carpobrotus edulis* L.) as well as cultivated (*Trifolium alexandrinum* L., *Beta vulgaris* L.) plants. Colonies of RMO (*Azospirillum brasilense*, *Enterobacter agglomerans* and *Klebsiella pneumoniae*) nicely developed on surface-inoculated agar plates prepared from crude and diluted juice of *M. crystallinum* (ice plant). Furthermore, hundreds of RMO colonies developed on various standard culture media were replicated (>90%) on agar plates of different plant juices. RMO cells grew nicely in liquid ice plant juice, with doubling times comparable to those grown in the reference culture medium. RMO populations resident in various host plants were able to develop on culture media prepared from homologous and heterologous juices. The application of a thin semi-solid overlay agar on the surfaces of inoculated agar plates significantly increased the recovery of micro-colonies on agar plates, particularly those prepared from plant juices.

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2090-1232

Peer review under responsibility of Cairo University.
doi:10.1016/j.jare.2011.03.002

Production and hosting by Elsevier

Introduction

Increasing the cultivability of RMO under laboratory conditions represents a challenge to specialists in the field. Cultivation on laboratory media has selective effects, and thus yields results that are not representative of the whole microbial community [1]. RMO communities develop in concert with the plant roots, and are, as well, framed by the background and bulk soil community [2]. This has affected the continuing efforts to formulate culture media for culturing RMO. The

addition of soil extract [3] to the generally-used nutrient agar [4] resulted in some progress, meeting some of the nutritional requirements of the soil but not of the plant. Including plant material in the composition of RMO culture media was sporadic, and originally experimented through the use of plant infusions and extracts as additional supplements for cultivation of plant/soil microorganisms. Tomato juice was included in culture media specific to *Lactobacillus acidophilus* and *Lactobacillus bulgaricus* [5,6]. The growth of lactic acid bacteria [7] and *Leuconostoc citrovorum* [8] was stimulated through enriching the selective culture medium with the juices of tomato, cabbage, grape and orange. Potato/carrot infusions are added to the culture media of yeast, moulds and fungi [9]. Cane juice was substituted for sucrose to exert selective power in LGP culture medium for enriching *Gluconactetobacter diazotrophicus* [10].

Here we present data on the sole use of plant juices, of desert plants in particular, as culture media for culturing the composite population of RMO.

Material and methods

Tested plants and preparation of plant juices

The major tested desert plant, *Mesembryanthemum crystallinum* (ice plant), grows on the sand dunes of north Sinai. The profuse and juicy biomass produced enough juice of suitable nutritional composition (Tables 1 and 3) to facilitate its use as a culture medium. Juices of other desert (*Zygophyllum album* L. and *Carpobrotus edulis* L.) and cultivated (*Trifolium alexandrinum* L. and *Beta vulgaris* L.) plants were also tested. The whole mature plant shoot, at flowering, was sliced and blended with the minimum amount of distilled water for ca. 5 min in a Waring blender. The resulting juice was coarse-filtered through cotton tissue and stored at −20 °C. The juice, as such or diluted with bi-distilled water (v/v), was exclusively used to prepare liquid and/or solid (2% agar, w/v) culture media, adjusted to pH 7.0 and autoclaved at 121 °C for 20 min. Chemical analyses of plant juices were carried out [11] and are presented in Tables 1 and 3.

Tested RMO and their reference culture media

Tested RMO were representatives of diazotrophs, *Azospirillum brasilense*, *Enterobacter agglomerans* and *Klebsiella pneumoniae*, originally isolated from desert plants [12,13], and routinely maintained on the combined carbon sources N-deficient medium, CCM [14]. The reference culture media used were nutrient agar [4], soil extract agar [3] as well as CCM.

The use of ice plant juice to prepare solid culture medium, and its ability to support growth of RMO colonies (cfu)

The first set of experiments dealt with the development of a selected number of RMO (diazotrophs) pure cultures. They were initially inoculated into liquid CCM medium (100 ml in 250 ml capacity flasks), supplemented with NH_4Cl (0.5 g^{-1}) and yeast extract (0.2 g^{-1}), then incubated in a rotary shaker (100 rpm) at 30 °C for 24 h. Serial dilutions prepared from the resulting liquid batch cultures were surface-inoculated on agar plates prepared from the ice plant juice (crude and further successive dilutions) and CCM for comparison. Inoculated plates were

Table 1 Chemical analyses[a] of tested plant juices.

Parameters	*M. crystallinum*	*T. alexandrinum*	*Z. album*	*C. edulis*
General				
EC (mmoh cm^{-1})	18.9	13.1	36.4	29.8
pH	6.03	6.10	5.48	5.81
Total nitrogen (%)	0.610	0.850	0.650	0.960
Organic carbon (%)	33.4	66.5	54.6	71.3
Cations (ppm)				
Ca^{++}	22	784	1636	1418
Mg^{++}	174	239	653	553
K^{+}	1853	2028	640	1338
Na^{+}	2714	782	4715	2852
Anions (ppm)				
HCO_3^-	4447	2135	1336	1958
Cl^-	3728	2847	9834	8165
SO_4^-	124	1435	2784	624
Soluble nutrients (ppm)				
Macro				
N	280	240	190	190
P	50	50	20	40
K	1800	2000	600	1300
Micro				
Zn	0.932	1.044	1.617	0.297
Fe	3.967	0.851	0.039	0.206
Mn	1.118	0.812	1.157	0.644
Cu	0.907	0.960	0.928	0.140
C/N ratio	55:1	78:1	84:1	74:1

[a] Cotteine et al. [11].

Table 2 Number of colonies replicated on agar plates of standard culture media and plant juices. Each set of experiments (A and B) was replicated 5 times.

Master plats (total No. of colonies)	Colonies replicated on secondary plates of tested culture media						
	Nutrient agar	Soil extract	CCM	*M. crystallinum* juice	*B. vulgaris* juice	*Z. album* juice	*C. edulis* juice
(A) Preliminary experiments (four plant juices)							
Soil extract (60)	51	58	57	50	30	36	6
CCM (21)	18	16	20	20	17	8	3
M. crystallinum (45)	38	44	41	43	34	20	8
B. vulgaris juice (19)	17	19	17	19	19	13	3
(B) Confirmatory experiments (two plant juices)[a]							
Soil extract (111)	18.2	21.2	18.1	19.4	18.2		
CCM (98)	15.8	17.0	17.0	16.8	16.4		
M. crystallinum juice (100)	13.5	15.2	15.2	16.2	13.8		
B. vulgaris juice (131)	18.2	18.5	20.8	21.2	21.7		

[a] ANOVA analyses was carried out for this particular sub-set of the experiments; each figure represent the mean value of five replicates; no significant differences were attributed to master plates, replicated secondary plates and one-way interaction. L.S.D. (at 0.05) = 14.8.

Table 3 Amino acids contents in the crude juice of *M. crystallinum*.

Amino acid	μg l^{-1}	Amino acid	μg l^{-1}
Aspartic	270	Proline	200
Threonine	140	Glycine	160
Serine	130	Alanine	250
Glutamic	470	Cysteine	100
Valine	250	Phenylalanine	130
Methionine	40	Histidine	210
Isoleucine	140	Lysine	180
Leucine	220	Arginine	360

incubated at 30 °C for 2–5 days and colony forming units (cfu) were counted.

The second set of experiments tested the ability of agar plates prepared from plant juices to replicate and support the growth of a wide array of RMO colonies originally developed on agar plates of reference culture media. For this purpose, RMO associated to roots of maize and sugar beet were cultured, using the surface-inoculation method and agar plates prepared from tested reference culture media [13]. The conventional replica technique of Lederberg and Lederberg [15] was adjusted and employed. Hundreds of 72-h-old RMO colonies developed on master agar plates, those prepared from all tested reference culture media with an average of 20–50 cfu $plate^{-1}$, were progressively stamped (5–7 times) onto agar plates of the tested plant juices. During a week of incubation at 30 °C, the successfully replicated colonies were monitored on the various combinations of plant juice agar plates and percentage recovery was calculated.

The use of ice plant juice as liquid culture medium for biomass production of RMO

The growth of tested RMO was tested in liquid culture media based on either crude ice plant juice or its successive dilutions. For comparison, liquid CCM (supplemented with 0.5 g^{-1} NH_4Cl and 0.2 g^{-1} yeast extract) was included. The liquid culture media were prepared (100 ml in 250 ml capacity Erlenmeyer flasks), inoculated with tested strains (2%, v/v), and incubated at 30 °C in a rotary shaker (100 rpm) for 7 days. Periodic samples were surface plated, in duplicate, for cfu counting; agar plates were prepared from both solid ice plant juice-based medium (crude juice diluted to 1:40 with distilled water, v/v) and solid CCM. Growth curves were plotted and doubling times were calculated [16]: Growth rate (K) = Log N_t − Log N_0/Log 2 (T_t − T_0); doubling time (dt) = 1/K, where N_0 = viable cell contents at T_0, T_0 = time at the beginning of determination, N_t = viable cell contents at T_t, T_t = time of determination.

The use of ice plant juice as solid culture medium for cfu counting of in situ RMO associated to various host plants

The rhizosphere of four host desert plants (*Hordeum murinum, M. crystallinum, Z. album* and *Stipagrostis scoparia*) and one cultivated Nile valley crop (*Hordeum vulgare*) was examined. Total RMO in the ecto- and endo-rhizospheres were determined using the ice plant juice (crude juice diluted 1:40 distilled water, v/v)-based agar medium and were compared with the reference media of nutrient agar, soil extract agar CCM. Ecto-rhizosphere samples were prepared [13] by transferring sufficient portions of root systems with closely adhering soil into sampling bottles containing the basal salt solution of CCM, as diluent. Bottles were shaken for 30 min and serial dilutions were prepared. The endo-rhizosphere samples were prepared [17] by washing another set of roots with tap water, then with 95% ethanol for 5–10 s, followed by 3% sodium hypochlorite for 1.5 h. Surface sterilized roots were then thoroughly washed with sterile water and crushed for 5 min in a Waring blender with adequate volume of basal salts of CCM medium. Further serial dilutions were prepared, and suitable dilutions of both spheres were surface-inoculated on agar plates prepared from all tested culture media. Incubation took place at 30 °C for 2–7 days and cfu were counted. Dry weights for suspended roots (80 °C) and rhizosphere soil (105 °C) were determined.

The use of homologous and heterologous plant juices for culturing RMO populations associated with plant roots

In addition to *M. crystallinum*, three more juicy plants (*C. edulis*, *Z. album* and *T. alexandrinum*) were evaluated for their

juices as culture media for RMO (Table 1). Obtained plant juices (crude juice diluted to 1:40, v/v) were used to prepare plating agar media. Making use of the surface–inoculated plates technique, RMO populations in the various root spheres, developed on homologous and heterologous plant juice-based culture media compared to all the tested reference culture media, were estimated in terms of cfu.

Modifying in situ culturing techniques of RMO

RMO of *T. alexandrinum* were assayed on culture agar plates of the homologous plant juice (diluted 1:40) as well as nutrient and soil extract agar. The culturing method was further modified by trying to adjust the oxygen diffusion at the surfaces of inoculated agar plates. Compared to the conventional surface-agar plate (method 1), a thin layer (2 ml) of semi-solid agar (0.6% agar) of the corresponding medium is overlaid on the agar surfaces, just after surface-inoculation and 30 min of surface drying (method 2). A third set of plates was prepared where the inoculum is mixed directly with the 2 ml overlay semi-solid agar medium prior to pouring onto agar surfaces. The developing cfu were assayed during a week of incubation at 30 °C, and differences attributable to culturing methods as well as culture media were statistically analyzed.

Data obtained throughout were statistically analyzed using STATISTICA 6.0 (StatSoft, Inc., Tulsa, USA). Analysis of variance (ANOVA) was used to examine the independent effects as well as possible interactions.

Results

The ice plant juice agar fully supports cfu development of RMO isolates

Colony forming units (cfu) of *K. pneumoniae* progressively developed on the crude juice, as well as its dilutions, with numbers comparable to those developed on CCM. Colonies of *A. brasilense* and *E. agglomerans* required the dilution of the crude juice. Further dilution of the juice (up to 1:50) did not affect cfu development (Fig. 1).

The suitability of plant juice-based culture media was not limited to the few tested RMO representatives but further extended to the wide spectrum of RMO populations. The conventional replica technique [15] was adjusted to reproduce RMO colonies developed on master agar plates of the tested reference culture media onto agar plates of plant juices. Preliminary experiments (Table 2A) significantly favoured the juices of *M. crystallinum* and *B. vulgaris*. Juices of *Z. album* and *C. edulis* replicated only 3–62% of colonies, possibly because of their higher content of total salts, Na, Ca and Cl, and/or lower values of N, P and K (Table 1). Further experiments (Table 2B) confirmed that juices of *M. crystallinum* and *B. vulgaris* successfully replicated, >90–100%, several hundreds of RMO colonies developed in reference culture media; the differences were not statistically significant.

The ice plant juice liquid culture medium supports cell growth and biomass production of RMO

Growth of RMO isolates was tested in liquid culture media based on either crude ice plant juice or its successive dilutions. Cells nicely developed in the plant juice batch cultures with a pattern very comparable to CCM; calculated doubling times were alike (Fig. 2). While *K. pneumoniae* favoured the growth on the crude juice, diluting the juice satisfied the requirement of *E. agglomerans* and *A. brasilense* (data not shown). Further dilutions of the crude juice (>1:20) slowed the cell growth of *E. agglomerans* and *K. pneumoniae* and affected *A. brasilense* severely.

The ice plant juice agar supported culturing of RMO associated with plant roots

The ice plant juice-based agar medium (diluted 1:40, v/v) was compared to nutrient agar, soil extract agar and CCM. The ecto-rhizosphere and endo-rhizosphere samples of desert

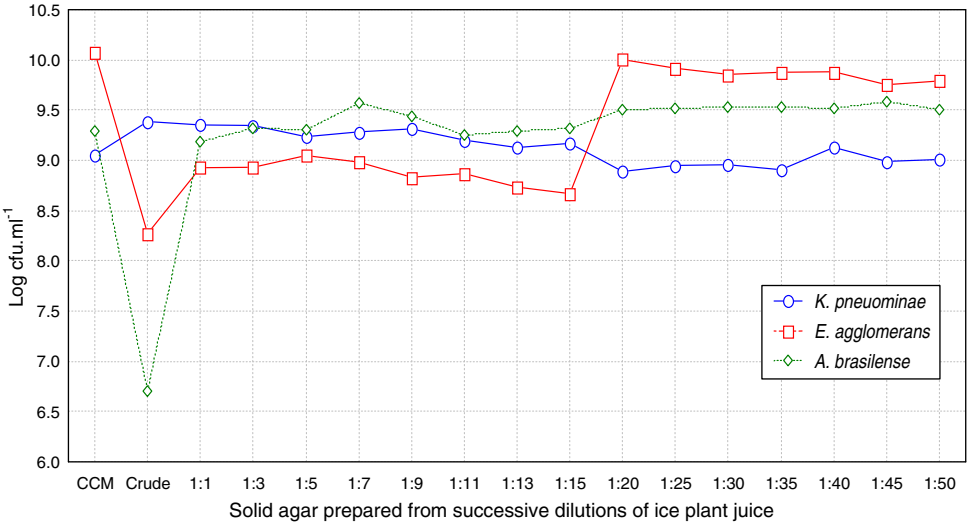

Fig. 1 Development of *K. pneumoniae*, *E. agglomerans* and *A. brasilense* on surfaces of agar plates prepared from ice plant (*M. crystallinum*) juice, at various concentrations (crude and diluted with water v/v, 1:1 up to 1:50); the reference CCM was included for comparison. Each point represents average of three replicates.

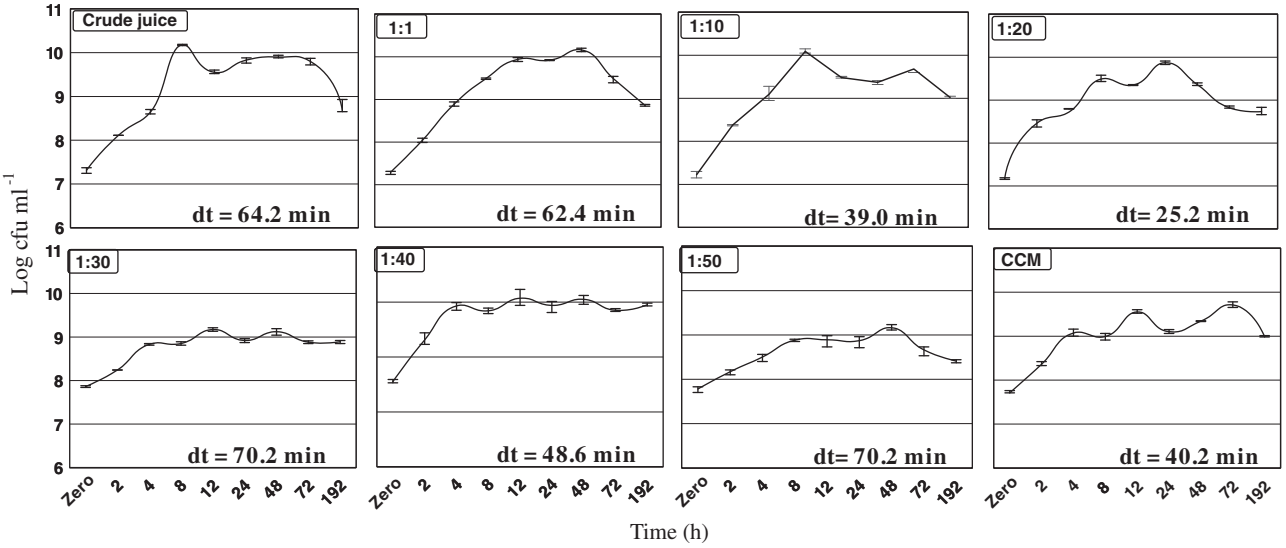

Fig. 2 Growth performance of *E. agglomerans* grown in batch cultures prepared from successive dilutions of ice plant juice, and plated on ice plant juice (dilution 1:40) agar medium as well as CCM. Inserted are the ice plant juice dilutions of the prepared liquid batch culture (crude juice, and dilutions 1:1, 1:10, 1:20, 1:30, 1:40, 1:50) and the doubling time (dt).

plants (*M. crystallinum*, *Z. album*, *S. scoparia* and *H. murinum*) and cultivated *H. vulgare* were assayed for total RMO. ANOVA analysis, two-way interactions, indicated highest RMO colonization in the ecto-rhizosphere. They developed comparable populations on plant juice, nutrient agar and CCM media but not on soil extract agar. RMO in the endo-rhizosphere were particularly supported with the plant juice-based culture media, while those in the rhizosphere developed the highest populations on the soil extract agar. Three-ways interactions indicated that the plant juice agar medium favoured RMO in the endorhizosphere and ectorhizosphere of most tested plants (Fig. 3). The salt accumulating *Z. album* accommodated the lowest populations of endophytes. In general, the rhizosphere soil recovered the lowest population except for the soil adjacent to the ice plant because its network of shoots above and below ground do enrich the soil and extend the boundaries of the rhizosphere beyond the commonly-accepted zone.

RMO develop on agar plates prepared from homologous and heterologous plant juices

Agar plates were prepared from the different juices (the crude and its further dilutions) of *M. crystallinum*, *C. edulis*, *Z. album* and *T. alexandrinum*. RMO populations in root spheres of such plants were estimated in terms of cfu developed on homologous and heterologous plant juices as well as the reference culture media. ANOVA analysis indicated the significance of independent effects of host plant, root sphere and type of culture media. In general, RMO nicely developed on homologous plant juice-based culture media, provided the crude juices

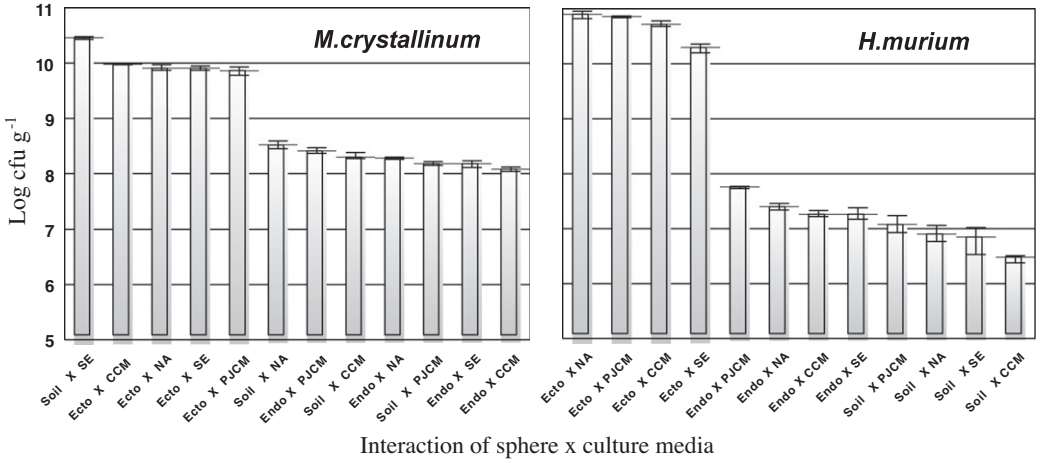

Fig. 3 Ranked RMO culturable populations in soil, ecto- and endo-rhizospheres of *M. crystallinum* and *H. murium* developed on agar plates of reference media (soil extract, SE; nutrient agar, NA; CCM) compared to the ice plant juice culture medium (PJCM). Data for other host plants are not shown.

had been diluted (1:10 for *Z. album* and *T. alexandrinum*, and 1:20 for *M. crystallinum*).

Two-way interactions indicated the significant effect of the plant juice origin (Fig. 4); the juice of *T. alexandrinum*, followed by *M. crystallinum*, supported the highest RMO populations colonizing the endo-rhizosphere, not only in homologous roots but also those nesting in all heterologous plant roots. The ecto-rhizosphere is significantly the richer; RMO did increase with the successive dilutions of plant juices. Statistical analysis indicated that all tested plant juices, aside from *C. edulis*, were able to support RMO in root spheres indiscriminately.

In another set of experiments, RMO of *T. alexandrinum* were assayed on culture agar plates of the homologous plant juice (diluted 1:40) as well as nutrient and soil extract agar. The culturing method was further modified by trying to adjust the oxygen diffusion at the surfaces of inoculated agar plates. A thin layer of semi-solid agar of the corresponding medium, with or without inoculum, was overlaid on the agar surface. cfu were statistically the lowest on nutrient agar and the highest on plant juice. The application of a thin layer of overlay semi-solid agar on top of the agar surface, post surface inoculation, did significantly increase micro-colonies, particularly on the plant juice agar plates (Fig. 5).

Discussion

The plant-soil system is a busy forum for multiple intercommunicating parties including microorganisms. The microbial communities in the rhizosphere are primarily non-specific and are selected through a combination of the available bulk soil microbial pool, plant species and environmental conditions [2]. The structure of rhizospheric microorganisms (RMO) is greatly determined by plant species [20], plant genotype [21], and plant nutrient status [22]. The orchestral effect of plant through root exudates is very well documented by the accumulating research on root exudates and their print on the structure of the rhizosphere microflora [23]. It is reported that plants release >20% of photosynthetically assimilated carbon in the form of carbohydrates, organic acids, amino acids and amides, vitamins and other compounds [24–26].

With the development of higher genetic diversity of RMO, measured as bands in de-naturing gradient gel electrophoresis (DGGE) [27,28], increasing culturability of RMO under labo-

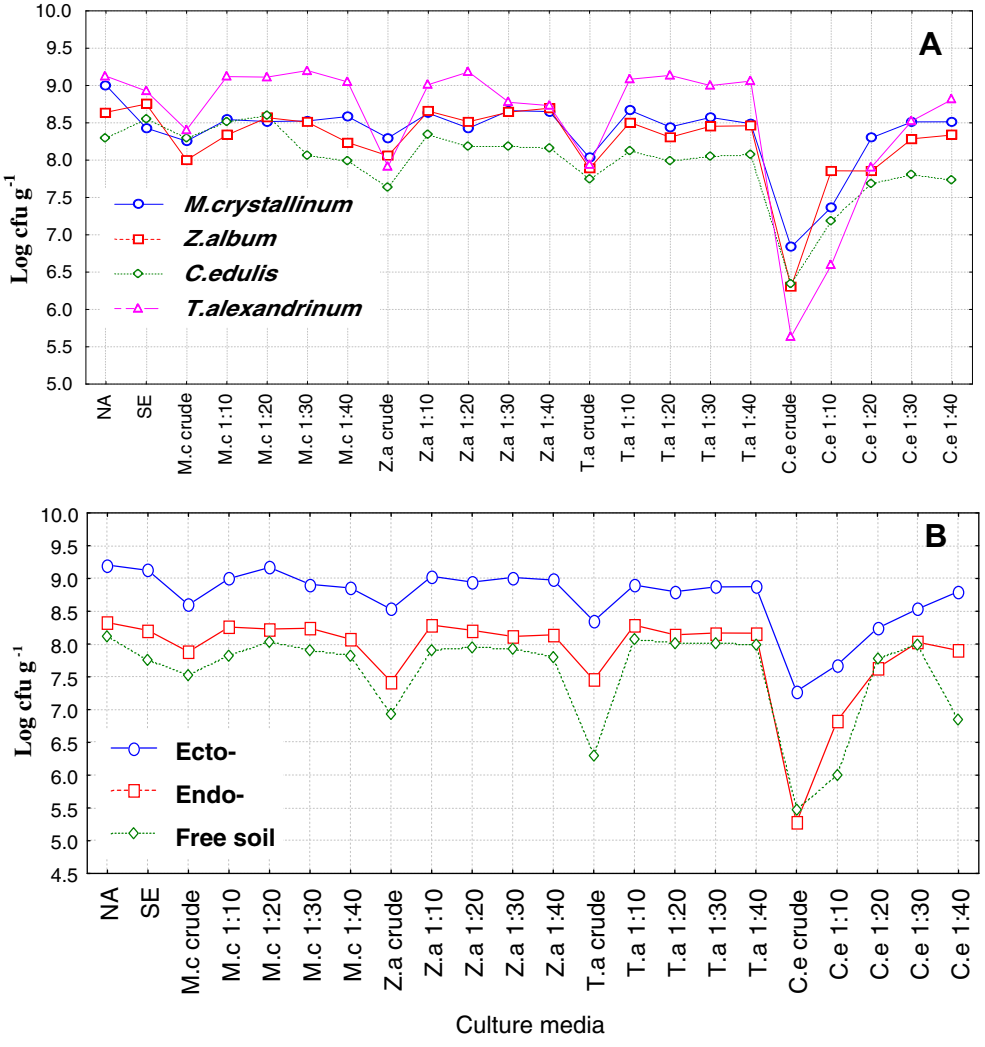

Fig. 4 RMO-culturable populations of tested host plants developed on agar plates prepared from reference media as well as homologous and heterologous plant juices, as affected by the tested host plant (two-way interaction, A) and the root sphere (two-way interaction, B).

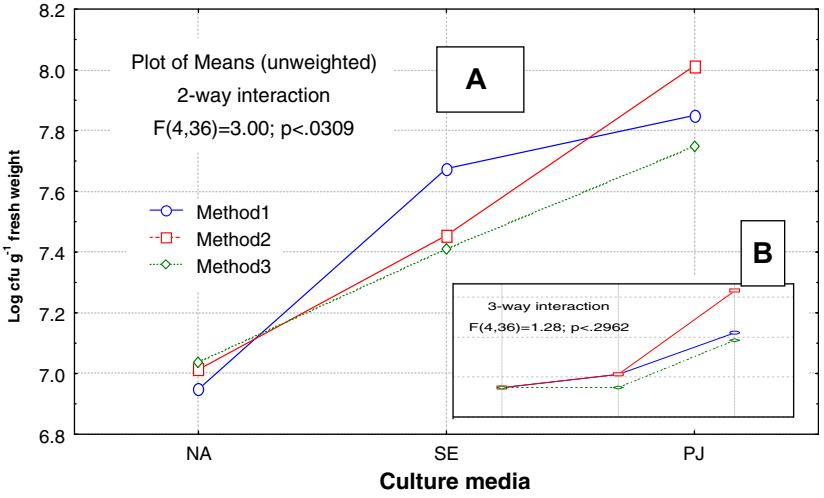

Fig. 5 (A) Total numbers of colonies (macro-and micro-) developed on agar surfaces of various culture agar media (nutrient agar, NA; soil extract, SE; plant juice, PJ) applying the conventional surface-inoculated plates (method 1), overlaying a thin layer (2 ml) of semi-solid agar (0.6%) of the corresponding medium on agar surfaces (method 2), and mixing the inoculum directly with the 2 ml semi-solid agar just prior overlaying on agar surfaces (method 3). (B) Progressive development of micro-colonies (inserted) developed on the tested three culture media by using the above-mentioned three methods of culturing.

ratory conditions is a challenge to specialists in the field because cultivation on laboratory media has selective effects, and thus yields results that are not representative of the whole microbial community [1]. Therefore, attempts to further enrich RMO culture media continue. One approach was the addition of soil extract to the generally used culture media such as nutrient agar. This resulted in some progress, meeting some of the nutritional requirements of the soil but not fully considering the plant effect. Further improvements in the cultivation and growth of microorganisms associated with plants were substantially achieved by the inclusion of plant infusions and extracts, e.g. tomato, cabbage, grape, orange and sugarcane, in their selective and synthetic culture media [5–8,10]. Amino acids and vitamins, as defined growth factors, were also added to the culture media. However, the supplements mentioned above might partially be of great assistance, if the complex chemical composition of the root exudates is taken into consideration. It is well established that plant roots exude a large amount and a complex assortment of organic compounds into the nearby soil. They vary in quantity and quality with plant species, genotype, age, physiological status, root morphology, location along the root, and the root environment including the presence of solid matrix, soil organisms and water [29–34]. The development of plant roots as well as of many biotic interactions in the rhizosphere involves chemical signals in response to environmental cues that induce specific responses (quorum sensing) including pathogenesis, release of extracellular enzymes, antibiotic production, biofilm formation, symbiosis initiation, and chemotactic motility [35,36]. Therefore, plants have the ability to affect the density-dependent behaviors of RMO by secreting such quorum-sensing mimic compounds or interference compounds [37]. Reciprocally, the microbial community has an elaborate and varied repertoire of signaling mechanisms that can affect plants as well. For example, bacteria can sense and respond to phytohormones from plants and release hormone analogs, resulting in either positive (e.g. plant growth promoting bacteria) or negative (e.g. deleterious rhizobacteria or pathogenic bacteria) effects [38].

To satisfy some of the RMO requirements provided by the interacting plant, the idea of including plant juices into culture media should be further intensified and researched. Here, we demonstrate that crude plant juices of almost all the tested plants (Table 2) are rather rich in nutrients that successfully and solely support the RMO growth, even to the extent that they are required to be further diluted for better growth and more applicability. It is calculated that an ice plant dilution of 1:40 contains ca. 0.8% organic carbon, compared to 0.3% in the synthetic and defined reference CCM culture medium [39], thus satisfying the needs of RMO. Both the ice plant and the berseem clover are favored because of their juicy nature (26–66% juice extraction). Chemical analyses (Table 1) support their superiority because of one or more of the following: higher pH (> pH 6), richness in carbon and nitrogen compounds, soluble macro-(N, P and/or K) and/or micro-(in particular Fe) nutrients. Amino acids as growth factors do present in the tested plant juices, e.g. the ice plant contained 16 amino acids with concentrations ranging from 100 to 400 $\mu g\,l^{-1}$ (Table 3). On the other hand, crude juices of *C. edulis* and *Z. album* are not ideal, and need to be modified because of their possible high content of salts, Na, Cl and/or SO_4, together with lower pH and Fe.

The tested juices of *M. crystallinum* and *T. alexandrinum* were of a promiscuous nature and were able to support the general development of RMO associated with a variety of tested host plants. This supports the idea that on a coarse taxonomic scale, there is some degree of commonality in the bacterial composition of rhizosphere communities of many plants, ignoring a certain degree of specificity in the selection of these communities [2]. Under the effect of plant exudates, conventional analyses [40,41] and DGGE analysis of 16S rRNA [23] suggested a strong shift from a highly diverse to a selected population.

cfu developed on tested reference culture media were successfully and efficiently replicated on plant juice agar plates

(Fig. 6). Moreover, cfu on plant juice agar plates were rather confined and non-slimy, eliminating the running together of colonies that cover up micro-colonies (μCo, < 1 mm dia discriminated with 40× magnification). This might support specific efforts to culture the unculturable RMO by further supporting the recovery of micro-colonies via dilution of nutrients present in the reference culture media [18] and/or adjustments of the in-vitro atmosphere of the surroundings [19]. On RMO plates counting in the present study, such micro-colonies were particularly distinguished, with higher incidence on the agar plates of plant juices. Additional significant increases in their numbers were achieved through the application of a thin layer of semi-solid agar overlaid on the surface-inoculated plates, particularly those of plant juices (Fig. 5).

The very significant richness of the ecto-rhizosphere of all tested host plants, using the plant-juice based culture media, supports the concept of the ''rhizosphere effect''. This agrees with the previously reported phylogenetic and functional composition of rhizosphere microbial communities. It is the net result of the plant interweaving with the indigenous soil community. Interacting factors include, in addition to the aforementioned plant exudates, flux of soluble salts into the rhizosphere under the effect of transpiration-driven movement of water, nutrient ion uptake and diffusional movement by roots creating zones of nutrient depletion, diurnal water potential fluctuations in the soil adjacent to roots [42,43], creation of zones of low O_2 concentrations [44], and change of the pH of the rhizosphere [45]. Such fluctuations are considered as a critical environmental characteristic for selecting rhizosphere microbial communities in quantity (compositional) and quality (functional). Results of 16S rDNA were able to relate the phylogenetic diversity to function through conventional interpretation [2]. The compositional and functional characteristics of the rhizosphere microbial community are determined by the integrated biotic interactions occurring in the rhizosphere habitat together with environmental determinants operating on the bulk soil microorganisms.

In conclusion, the results demonstrated the successful use of plant juice-based culture media for RMO cultivation. However, the presented qualitative approach remains to be further investigated quantitatively, using DNA-based methods, to distinguish the phylogenetic differences between cfu developed on plant juices and on reference culture media, to ascertain whether new types of bacteria are possibly cultured. Towards more standardization, the effect of plant variety and growth stage, previously established with tomato plants and lactic acid bacteria [8], remains to be experimented and verified.

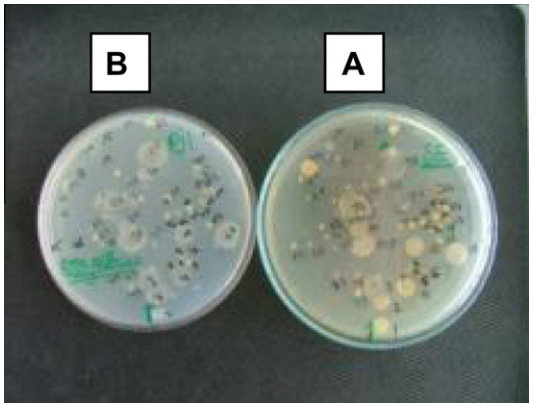

Fig. 6 Colonies developed on a master plate (A) of yeast extract (SE) successfully replicated (B) on the ice plant juice agar (PJ 1).

Acknowledgments

The authors would like to pay attribute to Cairo University on its centennial anniversary. We acknowledge the technical support of Gehan Yousssef of ARC Egypt and Frau Brigit Wernitz of IGZ, Germany. The financial support of Alexander von Humboldt Stiftung, Germany to N.A. Hegazi is appreciated. The present work was supported by the Research Grant BLAFE/FC31/3-94, of the ''Agrotechnologies based on biological nitrogen fixation for development of Sinai agriculture'' project.

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